9-(2´-Deoxy-2´-fluoro-ß-D-arabinofuranosyl) adenine: a potent antitrypanosomal adenosine analogue that circumvents transport-related drug resistance

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Abstract

Current chemotherapy against African sleeping sickness, a disease caused by the protozoan parasite *Trypanosoma brucei*, is limited by toxicity, inefficacy, and drug resistance. Nucleoside analogues have been successfully used to cure *T. brucei*-infected mice, but they all have the limitation of being taken up by the P2 nucleoside transporter, which, when mutated, is a common cause of multidrug resistance in *T. brucei*. We report here that adenine arabinoside (Ara-A) and the newly tested drug 9-(2′-Deoxy-2′-fluoro-ß-D-arabinofuranosyl) adenine (FANA-A) are instead taken up by the P1 nucleoside transporter, which is not associated with drug resistance. Similar to Ara-A, FANA-A was also found to be resistant to cleavage by methylthioadenosine phosphorylase, an enzyme that protects *T. brucei* against the antitrypanosomal effects of deoxyadenosine. Another important factor behind the selectivity of nucleoside analogues is how well they are phosphorylated within the cell. We found that the *T. brucei* adenosine kinase had a higher catalytic efficiency with FANA-A than the mammalian enzyme, and *T. brucei* cells treated with FANA-A accumulated high levels of FANA-A triphosphate, which even surpassed the level of ATP and led to cell cycle arrest, inhibition of DNA synthesis, and accumulation of DNA breaks. FANA-A inhibited nucleic acid biosynthesis and parasite proliferation with EC_{50} values in the low nanomolar range, whereas mammalian cell proliferation was inhibited in the micromolar range. Both Ara-A and FANA-A, in combination with deoxycoformycin, cured *T. brucei*-infected mice, but FANA-A did so at a 100 times lower dose than Ara-A.

44 **Introduction**

45

46 *Trypanosoma brucei* causes African sleeping sickness in humans and Nagana in cattle
47 and is transmitted between its mammalian hosts by tsetse flies (1, 2). The disease is
48 characterized by two stages. Initially the parasites are restricted to circulating in the blood
49 and lymph systems, but in the advanced stages of the disease they are also found in the
50 cerebrospinal fluid, which leads to coma and death if the patient is not treated. There is
51 no existing vaccine, and available treatments are specific to the disease stage and the
52 parasite subspecies. High toxicity, complex administration protocols, and drug resistance
53 all contribute to the urgent need for new therapies.

54

55 *T. brucei* lacks the pathway for *de novo* purine biosynthesis and is absolutely dependent
56 on exogenous purines that are taken up from the body fluids of its hosts (3). As a
57 consequence, the trypanosomes have evolved an array of efficient nucleoside and
58 nucleobase transporters for purine uptake (4). The efficient transporters and downstream
59 purine salvage enzymes can be exploited by developing antitrypanosomal substrate
60 analogues that utilize the powerful salvage systems in *T. brucei* and subsequently kill the
61 parasite by interfering with nucleotide-dependent reactions and/or nucleic acid
62 biosynthesis.

63

64 It is not only ribonucleosides that are salvaged by *T. brucei*. The parasite is also able to
65 salvage some deoxyribonucleosides, which are taken up by high-affinity nucleoside
66 transporters (5, 6) and subsequently phosphorylated by thymidine kinase and adenosine

kinase (7-10). Thymidine kinase phosphorylates thymidine and deoxyuridine (7), whereas adenosine kinase is able to salvage adenosine and deoxyadenosine (9). It has been shown that higher levels of dATP accumulate in *T. brucei* than in mammalian cells when treated with high concentrations of deoxyadenosine in the growth medium and that this accumulation is followed by *T. brucei* growth inhibition and lysis (11, 12). This effect is less prominent at lower concentrations of deoxyadenosine because *T. brucei* methylthioadenine phosphorylase (*Tb*MTAP) cleaves this substrate into adenine and deoxyribose-1-phosphate, and thereby rescues the parasite from the cytotoxicity of deoxyadenosine (11). At higher concentrations of deoxyadenosine, this protective system is inhibited by its reaction product, adenine, which allows the deoxynucleoside to be phosphorylated by adenosine kinase instead, leading to subsequent buildup of toxic levels of dATP in the cell (11). *Tb*MTAP is relevant for drug development because adenosine kinase substrate analogues need to be resistant to *Tb*MTAP cleavage in order to be effective against the parasites.

Nucleoside/deoxynucleoside analogs are commonly used as drugs for cancer therapy and antiviral treatments (13). One advantage of using nucleoside analogs against second-stage sleeping sickness is that many of them can cross the blood brain barrier via transporters used for the uptake of nucleosides needed by the brain (14). *T. brucei* bloodstream forms express two different purine nucleoside transporters, P1 and P2 (5, 15). The *T. brucei* P2 transporter has received much attention because of its role in multidrug resistance. It is involved in the uptake of diamidines and the melaminophenyl arsenicals, which are two of the main groups of antitrypanosomal agents known to be effective against *T. brucei* (5,

15). The P2 transporter is particularly important for the uptake of diaminazene aceturate (Berenil), a diamidine drug used against Nagana in cattle (16). Experience using this drug in cattle has shown that the trypanosomes rapidly become resistant (16), and specific resistance mutations in the P2 transporter have been experimentally verified (17). Moreover, the P2 transporter was shown to be the sole transporter for the furamidine trypanocides (18) that until recently were in clinical trials against sleeping sickness (19), and it has also been implicated as one of the main *T. brucei* transporters for melarsoprol and pentamidine (15, 20). A main reason for the easily acquired resistance is that the transporter is encoded by a single gene, *Tb*AT1 (5, 21). Mutations of this gene can mediate drug resistance with no major loss in purine nucleoside transport, which can still occur by the P1 transporter activity as well as by the various nucleobase transporters (22). In contrast, P1 nucleoside transporters are encoded by at least half a dozen genes spread throughout the genome, and the different genetic variants of the P1 transporter seem to have similar substrate specificities (23, 24). It is therefore unlikely that mutations or the deletion of only one of them could cause resistance to a nucleoside analogue.

Most of the antitrypanosomal adenosine/deoxyadenosine analogs developed so far, including cordycepin, 2-fluorocordycepin, and tubercidin, are mainly taken up by the P2 nucleoside transporter (25, 26), which constitutes a problem with respect to cross-resistance to existing therapies. Our aim in this study was to identify novel antitrypanosomal adenosine analogues that are taken up by the P1 transporter and, due to the many genetic variants, less likely to become ineffective as a result of gene mutation. Other criteria that need to be fulfilled for an efficient antitrypanosomal nucleoside

analogue exploiting the adenosine kinase-mediated salvage pathway are that it should be resistant to cleavage by *Tb*MTAP and it should be a good substrate of *T. brucei* adenosine kinase.

Material and methods

***Tb*MTAP cleavage assay.** *Tb*MTAP assays were performed as described previously with recombinantly expressed and purified enzyme (11). Briefly, the enzyme was diluted in a buffer containing 50 mM potassium acetate, 50 mM Tris-HCl pH 7.4, and 0.05% Tween-20 prior to use in the enzyme assay. Enzyme activity was checked by incubating the enzyme with the compound in question in the presence of 50 mM KH_2PO_4 (adjusted to pH 7.4 with KOH) for 30 min at 37°C. The reaction was stopped by incubation at 100°C for 2 min, and the reaction products were analyzed by HPLC as described (11) to determine if the compound had been cleaved by *Tb*MTAP. The amount of enzyme was adjusted for each substrate to give an assay that was linear over the 30-min incubation period.

***T. brucei* and mammalian cell culture.** *T. brucei* bloodstream forms were cultured at 37°C in 5% CO_2 and humidified atmosphere in HMI-9 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Thermo Fischer Scientific (Gibco), Waltham, MA, USA) (27). Several clonal *T. brucei brucei* cell lines were used, all derivatives of the Lister 427 strain (http://tryps.rockefeller.edu/trypsru2_pedigrees.html). This includes the Lister 427 strain in de Koning´s laboratory, TC221 in Hofer´s laboratory (a previous

136 gift from Prof. Opperdoes, Universite Catholique Louvain, Brussels, Belgium), and the
137 B48 multidrug-resistant cell line in which the *Tb*AT1/P2 gene first had been deleted by
138 homologous recombination (*Tb*AT1-KO) (28) and the knockout cells had subsequently
139 been subject to continuous exposure to increasing levels of pentamidine *in vitro* (29). Re-
140 introduction of the *Tb*AT1 gene in this line created B48 + P2 (17), resulting in a higher
141 and more stable expression of the P2 transporter, which appears to be downregulated *in*
142 *vitro* when it is expressed only from its original locus (18). Mouse BALB/3T3 fibroblasts
143 (ATCC no. CCL-163) were cultivated as monolayers at 37°C in 5% CO_2 and a
144 humidified atmosphere in Dulbecco's modified Eagle's medium (Sigma) supplemented
145 with 10% (v/v) heat-inactivated fetal bovine serum, glutamine (0.584 g/l), and 10 ml/l of
146 100× penicillin, and streptomycin (Thermo Fischer Scientific (Gibco)). Human
147 promyelocytic leukemia cells (HL-60 cells, ATTC number CCL-240) were grown as
148 suspensions of cells under similar conditions as the Balb/3T3 cells with the only
149 difference being that RPMI (Thermo Fischer Scientific (Gibco)) was used instead of
150 Dulbecco´s modified Eagle´s medium.
151
152 **Drug sensitivity assays.** FANA-A and other test compounds were serially diluted in
153 HMI-9 medium, and 100 µl was added to each well in two rows on a 96-well plate.
154 Cultures of bloodstream-form *T. brucei* were diluted to 2×10^5 cells/ml in HMI-9, and
155 100 µl of cells was added to all wells. The plate was incubated at 37°C and 5% CO_2 for
156 48 h, after which 20 µl of 0.5 mM of resazurin sodium salt (Sigma) in phosphate-buffered
157 saline (PBS) was added to each well and the plate was incubated for a further 24 h.
158 Fluorescence was measured with 540 nm excitation and 590 nm emission using an

159 Infinite M200 microplate reader (Tecan Group, Männedorf, Switzerland). EC_{50} values
160 were calculated after plotting the fluorescence against the test compound concentration
161 using a sigmoid curve with a variable slope (Prism 5.0, GraphPad software, LA Jolla,
162 CA, USA).
163
164 **Nucleotide pool measurement.** NTP and dNTP pools from *T. brucei* TC221 cells were
165 extracted as described previously (12). Briefly, *T. brucei* cells (10^8 cells) were lysed with
166 a trichloroacetic acid-$MgCl_2$ solution and centrifuged, and the supernatant was
167 subsequently extracted twice with freon-triocylamin to remove the trichloroacetic acid.
168 The resulting nucleotide extract (20 µl) was loaded onto a 100 mm × 4.6 mm ACE 3 AQ
169 column (Advanced Chromatography Technologies, Aberdeen, Scotland) run at 1 ml/min.
170 The mobile phase consisted of 11.3% (v/v) methanol, 80 mM KH_2PO_4, and 2 mM
171 tetrabutylammonium bisulfate, and the final solution was adjusted to pH 6 with KOH.
172 NTPs as well as FANA-A diphosphates and triphosphates were quantified by comparing
173 the peak heights to the results with a standard nucleotide solution. This HPLC protocol
174 was optimized for the determination of the FANA-A nucleotides and cellular NTPs,
175 whereas the cellular dNTPs were too low to be separated reliably from minor background
176 peaks.
177
178 **Measurement of [5,6-^{3}H]-uracil incorporation into *T. brucei* nucleic acids.**
179 Growing *T. brucei* TC221 (1.8–2 × 10^8 cells) were collected by centrifugation at 1,800 ×
180 *g* for 2 min and resuspended in 10 ml fresh medium. The resuspended cells were
181 incubated with 2 µM deoxycoformycin (dCF) for 30 min before treatment with various

concentrations of FANA-A for 1 h in the presence of 10 µCi [5,6-^{3}H]-uracil (Moravek Biochemicals Inc., Brea, CA). Extraction and precipitation of ^{3}H-labeled nucleic acids from *T. brucei* were performed as described, and the amount of radioactivity in the RNA and DNA was measured (9).

Assessing cell cycle progression in *T. brucei*. The cell cycle progression of bloodstream-form *T. brucei* (strain Lister 427) treated with FANA-A was assessed by visualizing the DNA in the nuclei and kinetoplasts using the fluorescent dye DAPI (4,6-diamidino-2-phenylindole) followed by microscopic analysis as previously described (30). Briefly, 50 µl of a trypanosome suspension (5×10^{5} cells/ml) was spread onto a glass microscope slide and left to dry before methanol fixation overnight at 20^{o}C. The slides were then rehydrated with 1 ml PBS for 10 min and then allowed to dry (but not totally). One drop of Vectashield antifade mounting medium with DAPI (Vector Laboratories Inc., USA) was added to the slides and spread out before covering with the coverslip. The slides were then viewed under UV light using a Zeiss Axioplan microscope with a Hamamatso digital camera and Openlab software. For each sample, 500 cells were counted and scored for their DNA configuration as 1N1K, 1N2K, 2N2K (Early), or 2N2K (Late) (where: N = nucleus; K = kinetoplast; 'Early' = no ingression furrow; and 'Late' = cell division has started with an obvious ingression furrow). Samples were taken at 0, 4, 8, 12, 16, and 24 hours, and untreated cultures were used as controls.

204 **Assessment of DNA integrity.** DNA fragmentation after exposure of *T. brucei*
205 bloodstream forms (Lister 427) to FANA-A (with or without dCF) was determined as
206 previously described (31). Drug-free and phleomycin (Sigma)-treated cells were used as
207 negative and positive controls, respectively. Briefly, the cell density was adjusted to 1 ×
208 10^6 cells/ml and the cells were incubated with or without the test compounds for 12 h.
209 The cells were fixed in 5 ml of 1% (w/v) paraformaldehyde in PBS, then centrifuged at
210 400 × *g* for 5 min and washed twice in 5 ml PBS. Finally, 5 ml of 70% ethanol was added
211 to the sample, which was kept at −20°C for at least 1 hour. The sample was then
212 centrifuged for 5 min at 400 × *g*. The pellets were resuspended in 1 ml of transporter
213 assay buffer (see below) and centrifuged for 5 min at 400 × *g*. The resuspension-
214 centrifugation step was performed twice to remove any residual ethanol. The presence of
215 DNA strand breaks in the FANA-A-treated trypanosomes was observed by flow
216 cytometry using the APO-BrdU TUNEL assay kit supplied by Life Technologies (catalog
217 no. A23210) according to the manufacturer's protocol where double-stranded breaks are
218 extended by terminal deoxynucleotide transferase in the presence of dNTPs and
219 bromodeoxyuridine (BrdU) triphosphate followed by immunolabeling of BrdU in DNA.
220
221 **Adenosine kinase assay.** Recombinant *T. brucei* adenosine kinase was expressed and
222 purified as described previously but using a concentration of 100 mM imidazole to elute
223 the protein in the Nickel-NTA agarose chromatography step (9). The enzyme assay
224 contained 3 mM ATP, 6.4 mM $MgCl_2$, 100 mM KCl, and 50 mM Tris-HCl pH 7.6.
225 Various concentrations of potassium phosphate (from a 100 mM KH_2PO_4-K_2HPO_4 pH
226 7.6 stock solution) and substrate (usually FANA-A) were included as described in the

figure legends. The enzyme (0.05 µg/reaction) was diluted prior to use in the assay with a buffer consisting of 50 mM Tris-HCl pH 7.6, 50 mM KCl, 0.1 mM dithiothreitol, and 0.05% (v/v) Tween-20. The 50 µl assay mixture was incubated at 37°C for 10 min and stopped by heating the sample for 2 min at 100°C. The separation of the reaction product from substrate, ATP, and other UV-absorbing compounds was performed by HPLC using a 50 mm × 2.1 mm Ultracore 2.5 SuperHexylPhenyl column (Advanced Chromatography Technologies) and a mobile phase containing 65 mM KH_2PO_4, 2 mM tetrabutylammonium bisulfate and 7% (v/v) methanol adjusted to pH 6 with KOH. The flow-rate was 0.4 ml/min and the injection volume 5 µl.

The short form of human adenosine kinase (32) was expressed, purified, and assayed in a similar way as described for the *T. brucei* enzyme except that the induction was performed for 10 hours at 30°C (instead of 3 hours at 37°C). The his-tag removal step was skipped in the purification protocol because it is known that the tag does not influence enzyme activity (32). The enzyme assay conditions were the same as for the *T. brucei* enzyme, and no adjustment to the amount of enzyme in micrograms was necessary because the molecular masses of the *T. brucei* and human (tagged) adenosine kinases are very similar in size (38 and 39 kDa, respectively). The bacteria expressing the human adenosine kinase (32) were part of a collection of mammalian adenosine kinases, bacteria and antibodies obtained as a kind gift from James Bibb at the University of Texas Southwestern Medical Center. All adenosine kinase assays were verified to be in the linear range with respect to time and protein amount.

Uptake assays. The assays were performed exactly as described previously (33, 34). Bloodstream forms of *T. brucei* cultures were harvested, washed with assay buffer (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM $CaCl_2$, 0.07 mM $MgSO_4$, 5.8 mM NaH_2PO_4, 0.3 mM $MgCl_2$, 23 mM $NaHCO_3$, and 14 mM glucose, pH 7.3) and resuspended at 10^8 cells/ml. The cells were incubated for 30 s with 0.1 µM [2,8-^{3}H]-adenosine in the presence or absence of competitive substrates. The uptake assay for the P1 transporter was performed with the *T. brucei* B48 knockout strain that lacks the P2 transporter. The uptake assay for the P2 transporter was performed with the same strain transfected with an expression construct carrying the P2 gene (B48 + P2), and in this case the experiment was performed in the presence of 1 mM inosine to saturate the P1 transporter. After the 30 s incubation with [^{3}H]-adenosine and competitors, the incubation was stopped with ice-cold unlabeled substrate (1 mM in assay buffer) and centrifuged through oil (13,000 × *g* for 1 min). This incubation time is well within the linear range of uptake as shown for both bloodstream and procyclic *T. brucei* (35). The cell pellet was transferred to a scintillation tube, and radioactivity was counted by a scintillation counter.

Adenosine deaminase assays. The presence of adenosine deaminase in the culture medium was measured by incubating the medium with up to 1 mM adenosine or FANA-A for up to 48 h at 37°C. The sample was centrifuged through a 3 kDa cutoff filter (Nanosep 3K Omega, PALL Corp., Port Washington, NY) and diluted 40 times in water. Subsequently, 5 µl of the diluted sample was analyzed by HPLC. The chromatography was performed on a 50 mm × 2.1 mm ACE Ultracore 2.5 SuperC18 column (Advanced Chromatography Technologies, Aberdeen, UK) using an aqueous mobile phase

containing 7% methanol and 42 mM ammonium acetate adjusted to pH 6 with acetic acid. The flow rate was 0.4 ml/min.

Mouse experiments. *T. brucei* TC221 bloodstream forms (20,000 cells) were injected intraperitoneally into black C57BL/6 mice. After confirmation of parasites in the blood (generally 3 days after injection), treatment started with an intraperitoneal injection of PBS buffer supplemented with 2 mg/kg FANA-A in combination with 0.25 mg/kg dCF. Similar experiments were also performed with 200 mg/kg Ara-AMP in combination with dCF. The doses were given twice daily on every alternate day for a total of 5 days (*i.e.* in the morning and afternoon on day 1, 3, and 5). Control mice were treated with dCF mixed in PBS or with only PBS. Blood samples were regularly collected from the tail and checked for the presence of *T. brucei* cells, and the mice were considered to be cured if they were parasite-free for 60 days after the last dose.

Results

Selection of adenosine analogues. One of the requirements for an adenosine kinase substrate analogue to be efficient against *T. brucei* is that it must be resistant to cleavage within the cell. Previous experiments have shown that adenine arabinoside (Ara-A) is a very poor substrate for the *Tb*MTAP enzyme that cleaves deoxyadenosine and that Ara-A sensitivity against *Tb*MTAP-knockdown trypanosomes was unaltered, indicating that its intracellular cleavage by *Tb*MTAP is insignificant (11). In addition to being cleavage resistant, Ara-A has good antitrypanosomal activity in the sub-micromolar range (9, 11).

295 Thus we tested the cleavage resistance and antitrypanosomal activity of other
296 deoxyadenosine and adenosine analogues having different 2′-substituents pointing in the
297 same direction as the 2′-OH group in Ara-A (Fig. 1). Two such compounds are
298 commercially available – FANA-A, which is a deoxyadenosine analogue with a fluorine
299 atom at this position, and 2′-C-methyladenosine, which is an adenosine analogue with a
300 methyl group at this position (Fig. 1). In addition, we also tested two variants of FANA-A
301 containing a halogen at the 2-position of the base to make them resistant to the adenosine
302 deaminase activity present in mammalian serum. These drugs included 2-fluoro-FANA-A
303 (2F-FANA-A), which was a kind gift from Jack Secrist at the Southern Research
304 Institute, and clofarabine, an anticancer agent used clinically (36). The advantage of the
305 deamination-resistant drugs is that they can be used as single agents in chemotherapy,
306 whereas other adenosine analogues generally need to be combined with adenosine
307 deaminase inhibitors such as dCF in order to be stable in circulation.
308
309 **Resistance to cleavage by *Tb*MTAP.** The selected compounds were tested in enzyme
310 assays with *Tb*MTAP. As shown in Table 1, all of the new compounds were even more
311 resistant to cleavage than Ara-A. In all four cases, the enzyme activity was below or very
312 close to the detection limit of the experiment. For comparison, the activity with the native
313 substrates methylthioadenosine, deoxyadenosine, and adenosine were more than 1000
314 times higher than any of the nucleoside analogues evaluated. We also tested the cleavage
315 of the different drugs in *T. brucei* cell extracts to determine if the drugs were recognized
316 by other nucleoside cleavage activities in *T. brucei*. The main purine nucleoside cleavage
317 activity in *T. brucei* cells is inosine-adenosine-guanosine nucleoside hydrolase (IAG-

NH), an enzyme that efficiently cleaves purine ribonucleosides but has very low activity on deoxyribonucleosides (37). The cleavage activity in the cell extract was below the detection limit for all of the nucleoside analogues tested, whereas the activity with adenosine, which was used as the positive control in the experiment, was ~1000 times higher than the detection limit (Table 1).

FANA-A is an efficient inhibitor of *T. brucei* proliferation. FANA-A was by far the *Tb*MTAP-resistant adenosine analogue with the greatest effect on *T. brucei* proliferation, with an EC_{50} value of 2.8 nM (Fig. 2A, Table 2). The EC_{50} value of 2′-C-methyladenosine was 300 nM, which is in a similar range as reported for Ara-A (9, 11). Both FANA-A and 2′-C-methyladenosine were also very selective, as indicated by the much higher EC_{50} values against the mammalian reference cell lines (Table 2), with FANA-A achieving selectivity indexes of 1900 and 790 (compared to Balb/3T3 and HL-60 cells, respectively). However, the addition of fluoro or chloro substituents at the 2-position of the nucleobase resulted in a reduced effectiveness against *T. brucei* proliferation. This effect can be attributed to these compounds being poor substrates of adenosine kinase, as predicted by previous molecular modeling studies with this enzyme (26). Clearly, the 2-position substitutions are poorly tolerated by adenosine kinase, and this is directly correlated to the size of the substituent because the chloro-substituted analogue clofarabine (2-chloro-FANA-A) displayed very low trypanocidal activity and the mammalian cells were much more sensitive than the trypanosomes.

340 **FANA-A is phosphorylated in *T. brucei* cells and inhibits DNA synthesis.** In
341 mammalian cells, it is well-established that fludarabine, clofarabine, cladribine, and other
342 nucleoside analogues lacking a 2′-OH group oriented as in ribonucleotides are
343 phosphorylated in the cell and subsequently inhibit DNA synthesis indirectly via
344 inhibition of ribonucleotide reductase and directly via inhibition of DNA polymerase
345 (38). Inhibition of ribonucleotide reductase is in most cases due to the fact that the
346 analogue mimics dATP as an overall negative regulator of activity, a type of regulation
347 that *T. brucei* lacks (12). Nucleotide pool analysis of FANA-A-treated *T. brucei* cells
348 showed that the parasites accumulated high levels of intracellular FANA-A 5′-
349 diphosphates and triphosphates, which increased above the level of ATP and became the
350 dominant nucleotides in the cell (Fig. 2B). The concomitant reduction in the ATP pool,
351 which was previously observed with deoxyadenosine-treated and Ara-A-treated
352 trypanosomes (11, 12), was only modest in the case of FANA-A, indicating that this is
353 probably not a significant factor contributing to the growth inhibition, which occurs at
354 much lower FANA-A concentrations than needed to reduce the ATP levels. A radioactive
355 tracer experiment showed that FANA-A blocked nucleic acid biosynthesis in the
356 parasites (Fig. 2C–D). When parasites were grown in the presence of ^{3}H-uracil and
357 various concentrations of FANA-A, the incorporation of radiolabel in DNA was strongly
358 inhibited, even at a FANA-A concentration of 0.1 μM (Fig. 2C). At higher concentrations
359 of the drug, RNA synthesis was also inhibited (Fig. 2D). In principle, the results in Fig.
360 2C-D could also be indirect effects caused by altered metabolism of the uracil that we
361 used as a probe or by FANA-ATP competing with ATP-dependent reactions in the cell.
362 However, the low concentrations of FANA-A and the short time (1 h) needed to give an

effect on DNA synthesis is a strong indication of specificity. For comparison, much higher concentrations were needed to obtain a large accumulation of FANA-ATP in the parasite (Fig. 2B). In addition, FANA-A does not resemble uracil in structure and the levels of UTP were normal in cells treated with the analogue (Fig. 2B). Unfortunately, the effect on the dNTP pools could not be determined accurately in these experiments because the FANA-A nucleotides interfered with the HPLC analysis. More experiments were therefore needed to confirm that the inhibition of DNA synthesis was a direct effect and not a cause of altered dNTP pools (see below).

We performed a series of experiments to assess whether FANA-A directly inhibits DNA synthesis (Fig. 3). Visual inspection of cultured *T. brucei* by fluorescence microscopy of DAPI-stained cells showed that the cell cycle distribution of trypanosomes treated with 0.2 μM FANA-A was similar to control cells during the initial hours after treatment. However, the treated cells gradually started to accumulate in the G1 stage with one nucleus and one kinetoplast (increasing from 60% of the cells in the G1 stage at the beginning of treatment to >90% after 24 h) (Fig. 3A). Concomitantly, the percentage of cells in all other cell cycle stages decreased. At the FANA-A concentration used, DNA synthesis was nearly completely inhibited (Fig. 2C) explaining why the change in cell cycle distribution takes 15 h even though a normal cell cycle is only 6 h. The accumulation of cells in the G1 stage means that they cannot pass the restriction point to reenter S-phase. For comparison, there was no change in cell cycle distribution in *T. brucei* treated with only dCF (Fig. S1). In order to determine if the altered cell cycle in FANA-A-treated *T. brucei* is due to DNA damage, we performed a TUNEL assay for the

386 detection of double-strand breaks. The FANA-A-treated parasites accumulated DNA
387 breaks, and this effect was even higher if the FANA-A was protected from deamination
388 by co-administration of dCF (Fig. 3B and Supplementary Fig. S2). The extent of DNA
389 breaks was almost as high as with phleomycin, which was used as a positive control in
390 the experiment. The induction of DNA breaks indicates that FANA-A interferes directly
391 with the accuracy of DNA replication.
392
393 **Adenosine kinase activity.** The first phosphorylation step is often rate limiting for the
394 formation of nucleoside analogue diphosphates and triphosphates in the cell (39). In order
395 to determine the reason for the selectivity of FANA-A against *T. brucei*, the recombinant
396 adenosine kinase activity with FANA-A as the substrate was compared to that of the
397 human adenosine kinase (Fig. 4). Mammalian and *T. brucei* adenosine kinases are known
398 to be dependent on phosphate ions for optimal enzyme activity when adenosine is the
399 substrate (9, 40). With FANA-A, the phosphate stimulation was fairly modest and was
400 saturated already at 5 mM phosphate with both enzymes (Fig. 4A). An experiment with
401 adenosine was also included for the human enzyme in order to highlight the difference in
402 activity compared to FANA-A. With adenosine, the effect of phosphate ions was much
403 stronger, and the activity continued to increase over the whole range of phosphate
404 concentrations tested (Fig. 4A). The main conclusion from a drug development
405 perspective is that FANA-A is a better substrate for the *T. brucei* adenosine kinase than
406 for the human enzyme and that this conclusion is valid regardless of the phosphate
407 concentration. The two enzymes were also studied using a fixed phosphate concentration
408 of 5 mM and varying substrate concentrations to obtain Michaelis–Menten parameters

(Fig. 4B). The *T. brucei* adenosine kinase activity had both a higher k_{cat} than the human enzyme (3.5 ± 0.3 s^{-1} vs. 0.99 ± 0.04 s^{-1}, calculated per polypeptide) and a slightly more favorable K_M (55 ± 16 µM vs. 96 ± 11 µM). 2F-FANA-A was also tested on the *T. brucei* enzyme, and it was found to be a much less favorable substrate (k_{cat}: 0.151 ± 0.006 s^{-1}, K_M: 84 ± 10 µM), explaining why the sensitivity of the trypanosome cells to this compound was two orders of magnitude lower than to FANA-A (Table 2).

FANA-A is taken up via the P1 nucleoside transporter family in *T. brucei*. Exhaustive characterization of purine transport activities in *T. brucei* has shown that the P1 and P2 transporters are the only transporters that facilitate the uptake of adenosine or its analogs in bloodstream forms of the parasite (4, 5, 25, 33). By comparing the FANA-A sensitivity of *T. brucei* cells (Lister 427-parent strain) with that of multidrug-resistant cells (strain B48) that lack both the P2 transporter and the high-affinity pentamidine transporter (HAPT) (29) and the same cells carrying a plasmid for stable expression of the P2 gene (strain B48 + P2) (17), it was possible to determine if the P2 nucleoside transporter plays a significant role in FANA-A uptake. As shown in Table 3, the sensitivity to pentamidine, which is known to be taken up by both the P2 nucleoside transporter and HAPT (later identified to be the aquaporin *Tb*AQP2 (41)), was dramatically reduced in the B48 strain and was partially restored when the P2 transporter was reintroduced. A complete reversion was not expected because the cells still lacked HAPT. In contrast, the sensitivity to FANA-A or 2F-FANA-A was not decreased in the multidrug-resistant cells, indicating that the P2 transporter is not important for the uptake of the drugs. Instead, a slight but significant increase in the sensitivity could be observed

in the P2 knockout compared to the parent strain, possibly because of compensatory upregulation of the P1 transporter in the absence of the P2 transporter. The reintroduction of the P2 transporter on a high-expression plasmid (B48 + P2 cells) caused only a ~30% increase in the FANA-A (+dCF) sensitivity compared to the B48 strain. This indicates that either FANA-A is a very poor substrate for the P2 transporter or uptake through the P1 transporter is already so efficient that it does not limit the antitrypanosomal effects of FANA-A.

Interestingly, Table 3 also shows that there was an order of magnitude difference in FANA-A sensitivity between experiments in the absence or presence of dCF. This indicates that the heat-inactivation procedure used to inactivate enzymes in the serum for the preparation of growth medium does not seem to be sufficient to disable all of the adenosine deaminase activity. This conclusion was verified in experiments where the deamination of deoxyadenosine in cell-free growth medium was checked by HPLC. Deoxyadenosine was nearly completely converted to deoxyinosine if incubated at 37°C for 48 h in growth medium containing heat-inactivated serum (Fig. 5A). In the presence of dCF, there was no deamination of either deoxyadenosine or FANA-A (Fig. 5A and B). Due to the lack of a reference compound for the deaminated form of FANA-A, we monitored the deamination by the disappearance of the FANA-A peak instead. The results shown in Fig. 5 are just a few examples from several experiments where the incubation time, substrate concentration, and/or source of serum was varied (commercially available heat-inactivated sera were also tested). A general conclusion was

454 that heat-inactivation only has a modest effect on adenosine deaminase. The deamination
455 was negligible in the first hours of incubation but became a severe problem after 24 h.
456
457 The roles of the P1 and P2 nucleoside transporters for FANA-A uptake were also tested
458 in assays for the ability of the drug to inhibit [^{3}H]-adenosine uptake in trypanosomes
459 (Table 4, Fig. 6). As expected, FANA-A was a good competitor for the P1 transporter
460 with a K_i value similar to the K_M of adenosine itself. The result with the P2 transporter
461 was much more surprising. The K_i value was 10 times lower than with adenosine,
462 indicating that it seems to be a substrate with superior binding affinity (with a difference
463 in Gibbs free energy of binding ΔG^0 of 5.7 kJ/mol), suggesting that a 2′-fluorine pointing
464 upwards in a Haworth projection actually contributes to the binding interactions with the
465 transporter, although a hydroxy moiety at that position is not favored (K_i = 1.9 µM for
466 Ara-A). It has previously been shown that a 'down'-directed hydroxyl group at the 2′
467 position is also not favored because deoxyadenosine has greater affinity compared to
468 adenosine (5) (Table 4). Thus we also tested the stereoisomer of FANA-A, 2′F-2′-
469 deoxyadenosine, and found that it had almost as high binding affinity as FANA-A (Table
470 4). A fluorine at the 2′-position, in either direction, seems consequently to be favorable
471 for high-affinity binding to the P2 transporter. In conclusion, FANA-A interacts with
472 both types of transporters efficiently, although the P1 transporter alone appears to be
473 sufficient for the antitrypanosomal effect.
474
475

***In vivo* experiments.** Black C57BL/6 mice were infected with *T. brucei* TC221 bloodstream forms, and treatment was initiated with the tested drug (Ara-AMP or FANA-A) injected intraperitoneally in combination with dCF as soon as the parasites were detectable in the blood. Because Ara-A has low aqueous solubility, it was given in the form of Ara-AMP in these experiments. Ara-AMP is a prodrug, which, upon dephosphorylation to Ara-A in the blood, is able to cross the cell membrane (42). As shown in Table 5, all the mice treated with any of the two drug combinations were cured. The level of parasites in the blood decreased below the detection limit within a day after the FANA-A treatment was initiated and remained the same during the rest of the treatment and for an additional 60 days of drug-free follow up, demonstrating that all of the mice were cured. Similar results were obtained for the Ara-AMP treatment except that it sometimes took two days for the parasite levels to go below the detection limit. No obvious side effects were observed with the FANA-A treatment, whereas the Ara-AMP treatment gave some weight loss that was recovered in all mice after the treatment was finished. On average, the weight loss in the Ara-AMP treated mice was a 13 ± 2% (standard error) difference between the starting weight and the lowest weight during the treatment. No weight loss was observed in the FANA-A-treated mice. Control groups, which received dCF or only PBS, were sacrificed three days after the treatment was initiated due to heavy parasite loads. Because the dosage protocol described here was 100% curative, it is likely that lower dosages or fewer administrations might still be effective, and the lower limits required for cure remain to be determined. Importantly, the mice treated with FANA-A did not suffer any overt toxic effects from the current protocol.

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499

500 **Discussion**

501

502 To date, the antitrypanosomal adenosine analogs developed against *T. brucei* are taken up
503 primarily by the P2 transporter (25, 26, 43). However, because mutations in this
504 transporter are strongly associated with resistance to current treatments (44-47), we
505 propose that adenosine analogs that are instead taken up by the P1 transporter, or by both
506 the P1 and P2 transporters, should be prioritized in the search for new agents against *T.*
507 *brucei*.. Here, we have identified FANA-A as an example of such an agent. FANA-A
508 inhibited *T. brucei* proliferation in the low nanomolar range, and multidrug-resistant
509 trypanosomes lacking the P2 transporter were equally sensitive to the drug as the parent
510 strain, showing that the P1 transporter alone is sufficient for the uptake of the drug. Yet,
511 the uptake assays showed that FANA-A very strongly inhibits adenosine uptake by both
512 the P1 and P2 transporters. FANA-A contains both the P2 binding $H_2N\text{-}C(R^1)\text{=}N\text{-}R^2$
513 'amidine motif' that is either incorporated into or attached to an aromatic ring (45, 48)
514 and the P1 binding motif, including the N(7) and N(3) atoms of the base as well as the 3′
515 and 5′-hydroxyl groups of the ribose (5, 23). Thus, FANA-A provides a perfect fit for the
516 recognition motifs of both *T. brucei* nucleoside transporters. The observation that the P1
517 and P2 transporters both display very high affinity for FANA-A is consistent with the P2
518 knockout experiment that shows that this transporter is not important for the trypanocidal
519 activity of FANA-A. One possibility could be that FANA-A efficiently binds to this
520 transporter but that its translocation rate is very slow (low V_{max}/K_M for the substrate)
521 possibly because of a slow off-rate linked to the unusually high binding affinity, but this

522 can only be assessed using radiolabeled FANA-A as the substrate, which was not
523 available. The alternative explanation is that uptake by the P1 transporters, which are
524 encoded by several genes, is already sufficient, with the toxicity being limited by the rate
525 of phosphorylation by adenosine kinase or the rate of incorporation into DNA, rather than
526 the rate of uptake.
527
528 The sensitivity of the parasites to FANA-A seems to be dependent on many factors,
529 including the efficiency of uptake and phosphorylation, resistance to cleavage, and effect
530 on DNA biosynthesis (Fig. 7). The affinity of FANA-A for the P1 transporter is as high
531 as that of adenosine, which is one of the main physiological substrates of this transporter,
532 and as mentioned above it is likely that the P2 transporter adds additional uptake
533 capacity. The initial phosphorylation of FANA-A, which is carried out by adenosine
534 kinase, is also efficient with a six times higher catalytic efficiency (k_{cat}/K_M) for the *T.*
535 *brucei* enzyme compared to the human enzyme, although it should be remembered that
536 phosphorylation of substrates in the cell is dependent on both the catalytic efficiency of
537 the enzyme and its physiological concentration and that mammalian cells also have
538 deoxycytidine kinase that can phosphorylate deoxyadenosine analogues in the cytosol
539 (39). The efficient uptake and phosphorylation of FANA-A was confirmed in *T. brucei*
540 cells, which accumulated higher concentrations of FANA-ATP than the endogenous level
541 of ATP. Another main factor behind the efficient accumulation of FANA-ATP is that
542 FANA-A is not recognized by *Tb*MTAP and other cleavage activities in the cell and is
543 thereby protected from degradation. The ultimate effect of FANA-A is that it interferes
544 with DNA synthesis and causes DNA breaks. For nucleoside analogues, this is a very

strong indication that they are incorporated into DNA. DNA breaks have previously been observed in mammalian cells treated with F-Ara-A, which was found to be incorporated into DNA where it acted as a structural chain terminator (36). In contrast to classical chain terminators, F-Ara-A does not lack the 3′-OH group. Instead, it is the structure of the DNA that is altered in such a way that the 3′-end cannot be extended, leading to breakage-sensitive single-stranded regions in the replicated DNA (36, 49). While delayed non-obligate chain terminators allow the DNA polymerase to add a few nucleotides after the incorporation site, F-Ara-A, clofarabine (which resembles FANA-A), and many other 2′-modified nucleoside analogues are pseudo-obligate, which means that they resemble classical terminators in that the 3′-end is not extended further (49, 50).

Experience so far has shown that most adenosine analogues tested cross the blood-brain barrier to varying degrees (51, 52), and Ara-A given intravenously has successfully been used to cure herpes simplex brain infections in humans (53). However, the neurological side effects reported for a few Ara-A-treated patients (54), and the need to use the phosphorylated form of the drug to make it soluble, limit its applicability and appear to make FANA-A a more interesting drug candidate. Neither FANA-A nor Ara-A can be used as single agents in therapy and need to be combined with dCF, which is also called pentostatin, in order to be protected from deamination by adenosine deaminase in the mammalian blood (Fig. 7). dCF is known to cross the blood-brain barrier in monkeys (55), making it suitable for use in second-stage sleeping sickness. A possible problem with dCF is that it has been reported to be teratogenic, but only if given on day 7–8 in pregnant mice (56). However, a later study that compared mice and rabbits found that this

568 was a mouse-specific effect, although this has to be verified in other animal models as
569 well if it is going to be used in pregnant sleeping sickness patients (57). Nevertheless, it is
570 still an advantage if FANA-A can be modified in a way that it can be used as a single
571 agent instead. One such modified analogue is 2F-FANA-A, which we found was 20 times
572 more selective against *T. brucei* than the mammalian fibroblasts used as reference.
573 However, this level of selectivity is quite modest compared to FANA-A, and we are
574 currently experimenting with other ways of making the molecule resistant to adenosine
575 deaminase. In conclusion, FANA-A is a promising candidate for further drug
576 development and demonstrates that nucleoside analogues can be created that are taken up
577 principally by the P1 nucleoside transporter, thereby avoiding the drug resistance
578 problems associated with uptake by the P2 nucleoside transporter.
579

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Figure legends.

Fig. 1. Deoxyadenosine and adenosine analogues containing 2′-substituents oriented as in Ara-A. The 2′position is highlighted in the left figure. Adenosine, which is not included in the figure, has its 2′-hydroxyl group pointing in the same direction as in 2′C-methyladenosine.

Fig. 2. Effect of FANA-A on *T. brucei* TC221 proliferation, nucleotide pools, and nucleic acid biosynthesis. (A) Inhibition of *T. brucei* TC221 proliferation as a function of FANA-A concentration. (B) Pools of ATP (■), FANA-A triphosphate (▽), FANA-A diphosphate (△), UTP (▼), and GTP (▲) in *T. brucei* cells treated with various concentrations of FANA-A. (C) The effect of FANA-A on DNA biosynthesis. (D) The effect of FANA-A on RNA biosynthesis. All experiments were performed with 2 μM dCF in the growth medium, and the results are based on at least three independent experiments with standard errors indicated.

Fig. 3. Cell cycle distribution and DNA fragmentation in FANA-A-treated *T. brucei* Lister 427 cells. (A) Cell cycle distribution in *T. brucei* treated with 0.2 μM FANA-A and 2 μM dCF (open symbols) as compared to untreated cells (filled symbols). Based on the number of nuclei (N) and kinetoplasts (K) and the presence of a furrow ingression, the cell cycle was divided into 1N1K (○, ●), 1N2K (△, ▲), early 2N2K (▽, ▼), and late 2N2K cells with initiated cytokinesis (□, ■). The ordinate is split into two segments to be able to enhance the lower part. (B) The percentage of DNA fragmentation as determined by the TUNEL assay on *T. brucei* grown in the absence (control) or presence of 2 μg/ml phleomycin (Phleo), 0.2 μM FANA-A, or a combination of 0.2 μM FANA-A and 2 μM

dCF. The results in A and B represent the average of two and three independent experiments, respectively, with indicated standard errors and *p*-values from a Student´s t-test where the results were compared to the untreated control cells (* $p < 0.05$, *** $p < 0.001$).

Fig. 4. *T. brucei* and human adenosine kinase activities. (A) Enzyme activity as a function of phosphate concentration with *T. brucei* and human adenosine kinases. The substrates were 100 µM FANA-A with the *T. brucei* adenosine kinase (■) and 100 µM FANA-A (□) or 100 µM adenosine (△) with the human adenosine kinase. (B) Enzyme activity as a function of the FANA-A concentration with *T. brucei* (■) and human (□) adenosine kinases. *T. brucei* adenosine kinase activity with 2F-FANA-A is shown for comparison (♦). All experiments were performed with 3 mM ATP as the co-substrate, and the graphs represent the average from at least three independent experiments with standard errors indicated.

Fig. 5. HPLC profiles showing the deamination of 1 mM deoxyadenosine (A) and FANA-A (B) when incubated in HMI-9 medium. The medium contained 10% heat-inactivated fetal bovine serum, and the measurement was performed after 48 h incubation at 37°C. The chromatograms are shown in a staggered view where the baselines are shifted vertically to fit several traces in each plot.

Fig. 6. Transport of [^{3}H]-adenosine by *T. brucei* bloodstream forms (A) Transport of 0.1 µM [^{3}H]-adenosine by the B48 cell line over 30 s, in the absence (first data point) or

presence of unlabelled adenosine (▲) or FANA-A (□) at the indicated concentrations. (B) Transport of adenosine in the presence of unlabelled adenosine (▲) or FANA-A (□). The experiments were performed in a similar way as in panel A, but using B48 cells expressing the P2 transporter (B48+P2), and the experiment was performed in the presence of 1 mM inosine for all data points in order to block the P1 transport activity. Both panels are representative of at least three independent experiments with standard errors indicated.

Fig. 7. FANA-A metabolism and mechanism of action in *T. brucei*. The P2 nucleoside transporter is not included in the figure because the P1 transporter is sufficient to give maximal growth inhibition by FANA-A. dCF, deoxycoformycin, ADA, adenosine deaminase, MTAP, methylthioadenosine phosphorylase.

Table 1. *Tb*MTAP activity with its native substrate methylthioadenosine and different nucleoside analogues used at 100 µM. Methylthioadenosine and adenosine were used as positive controls for the *Tb*MTAP and cell extract cleavage activities, respectively.

Substrate	***Tb*MTAP activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)**	**Cleavage in cell extracts ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)**
Methylthioadenosine	4.3 ± 0.2	*Not analyzed*
Deoxyadenosine	16.7 ± 0.7	*Not analyzed*
Adenosine	11.8 ± 0.3	0.16 ± 0.05
Ara-A	0.0027 ± 0.0004	<0.0002
FANA-A	0.0006 ± 0.0002	<0.0003
2F-FANA-A	<0.0002	<0.0002
Clofarabine	<0.00006	<0.0001
2′C-methyladenosine	0.0007 ± 0.00006	<0.0003

Table 2. Inhibition of *T. brucei* TC221, mouse Balb/3T3, and human HL-60 cell proliferation with adenosine analogues. FANA-A and 2′C-methyladenosine were given together with 2 µM deoxycoformycin (dCF).

Nucleoside analogue	***T. brucei* EC_{50} (µM)**	**BALB/3T3 EC_{50} (µM)**	**HL-60 EC_{50} (µM)**	**Selectivity Index**
FANA-A (+dCF)	0.0028 ± 0.0002	5.4 ± 1.2	2.2 ± 0.1	1900; 790
2F-FANA-A	0.56 ± 0.11	10 ± 5		18
Clofarabine	75 ± 17	1.24 ± 0.03		0.017
2′C-methyladenosine (+dCF)	0.30 ± 0.07	11 ± 7		37

Table 3. The effect of FANA-A and 2F-FANA-A on the proliferation of *T. brucei* Lister 427 (parent strain), P2 knockout cells (strain B48), *Tb*B48 cells complemented with the P2 nucleoside transporter (strain B48 + P2), and *T. brucei* strain TC221. Pentamidine was used as the positive control. P-values from Student´s t-tests were obtained by comparing B48 vs. Lister 427, and B48 + P2 vs. B48 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns – not significant).

Strain	**Pentamidine EC_{50} (µM)**	**FANA-A EC_{50} (µM)**	**FANA-A (+dCF) EC_{50} (µM)**	**2F-FANA-A EC_{50} (µM)**
Lister 427	0.0030 ± 0.0002	0.120 ± 0.001	0.014 ± 0.001	0.47 ± 0.09
B48	0.65 ± 0.03****	0.079 ± 0.005**	0.0072 ± 0.0004***	0.094 ± 0.016***
B48 + P2	0.049 ± 0.003****	0.060 ± 0.001*	0.0049 ± 0.0004*	0.11 ± 0.03 (ns)
TC221		0.25 ± 0.06	*See Table 2*	*See Table 2*

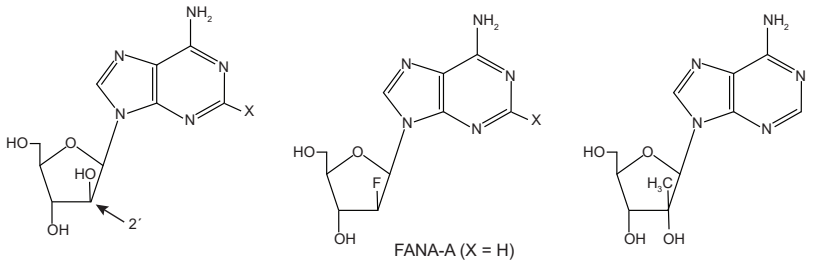
Table 4. Adenosine transporter assay.

Compound	**P1 transporter K_m or K_i (µM)**	**ΔG^0 kJ/mol**	**P2 transporter K_m or K_i (µM)**	**ΔG^0 kJ/mol**
Adenosine[1]	0.41 ± 0.08	−36.8	0.92 ± 0.06	−34.5
FANA-A	0.48 ± 0.11	−36.1	0.09 ± 0.02	−40.2
2´F-deoxyadenosine	0.06 ± 0.01	−41.1	0.19 ± 0.03	−38.3
Ara-A	ND		1.9 ± 0.5	−32.6
Deoxyadenosine[2]	0.19 ± 0.02	-38.3	0.23 0.04	-37.9

[1]K_M value for adenosine, the other values are K_i values (inhibition constants) determined by competition against [^{3}H]-adenosine transport.
[2]Values used from De Koning and Jarvis, 1999 (5), included for comparison.
ND, not determined.

Table 5. Effect of intraperitoneal FANA-A and Ara-AMP treatments on *T. brucei*-infected mice. The dose was given in the morning and evening on day 1, 3, and 5.

Drug	Dose (mg/kg) × 2 ×3	Cure rate
Ara-AMP (+dCF)	200 (+ 0.25 dCF)	10/10
FANA-A (+dCF)	200 (+ 0.25 dCF)	10/10
dCF	0.25	0/5
Only PBS	-	0/7



Ara-A (X = H)
F-Ara-A (X = F)

FANA-A (X = H)
2F-FANA-A (X = F)
Clofarabine (X = Cl)

2´C-methyladenosine

Figure 1

Antimicrobial Agents and Chemotherapy

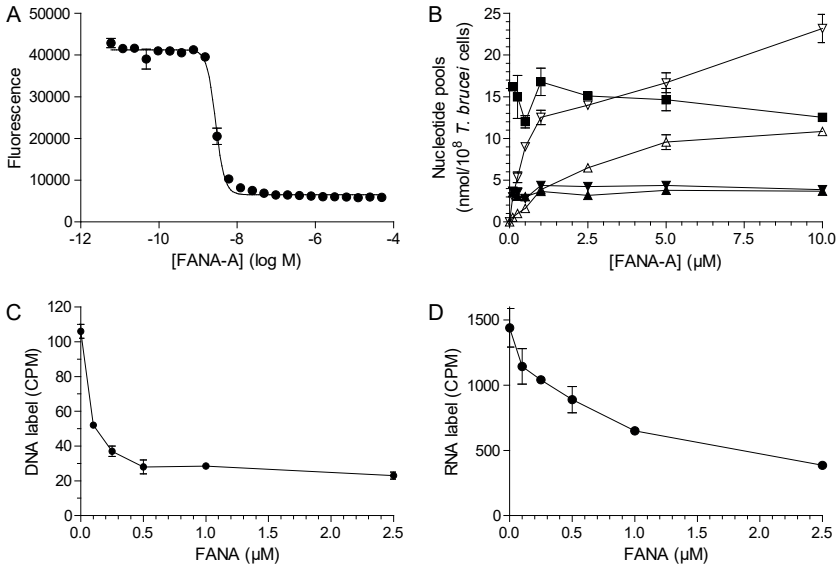


Figure 2

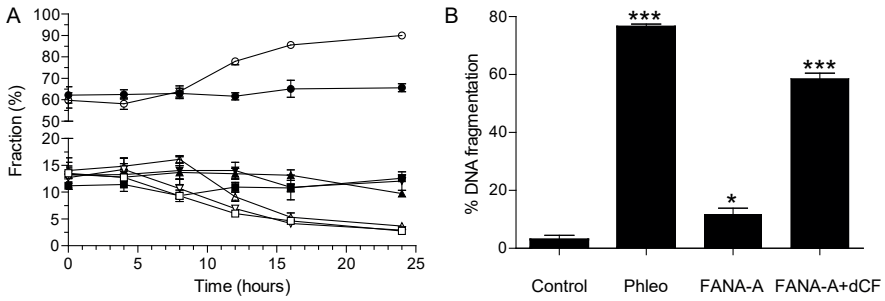


Figure 3

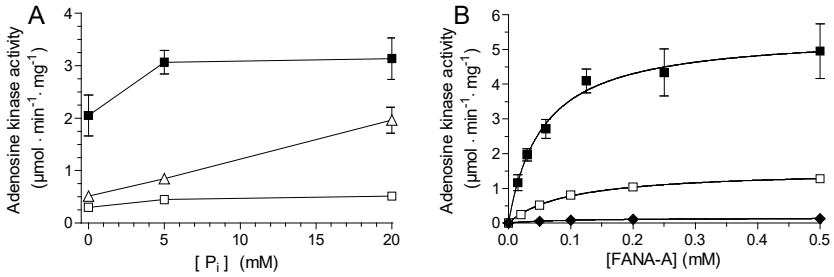


Figure 4

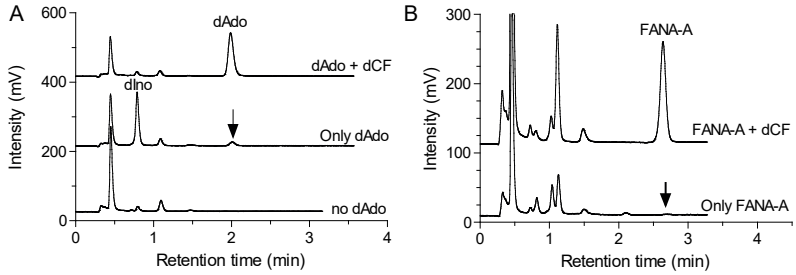


Figure 5

Antimicrobial Agents and Chemotherapy

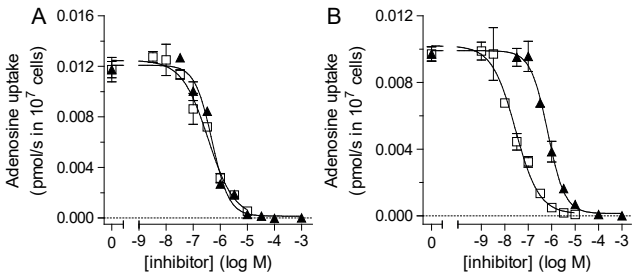


Figure 6

Antimicrobial Agents and Chemotherapy

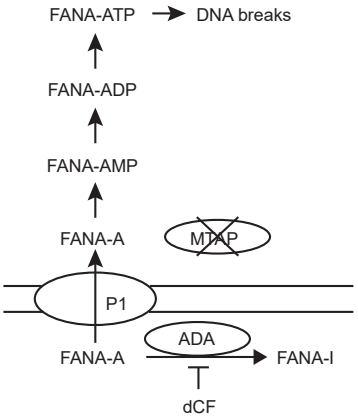


Figure 7

Antimicrobial Agents and Chemotherapy